

TRPA1 mediates trigeminal neuropathic pain in mice downstream of monocytes/macrophages and oxidative stress

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Despite intense investigation, the mechanisms of the different forms of trigeminal neuropathic pain remain substantially unidentified. The transient receptor potential ankyrin 1 channel (encoded by *TRPA1*) has been reported to contribute to allodynia or hyperalgesia in some neuropathic pain models, including those produced by sciatic nerve constriction. However, the role of TRPA1 and the processes that cause trigeminal pain-like behaviours from nerve insult are poorly understood. The role of TRPA1, monocytes and macrophages, and oxidative stress in pain-like behaviour evoked by the constriction of the infraorbital nerve in mice were explored. C57BL/6 and wild-type (*Trpa1*^{+/+}) mice that underwent constriction of the infraorbital nerve exhibited prolonged (20 days) non-evoked nociceptive behaviour and mechanical, cold and chemical hypersensitivity in comparison to sham-operated mice ($P < 0.05$ – $P < 0.001$). Both genetic deletion of *Trpa1* (*Trpa1*^{-/-}) and pharmacological blockade (HC-030031 and A-967079) abrogated pain-like behaviours (both $P < 0.001$), which were abated by the antioxidant, α -lipoic acid, and the nicotinamide adenine dinucleotide phosphate oxidase inhibitor, apocynin (both $P < 0.001$). Nociception and hypersensitivity evoked by constriction of the infraorbital nerve was associated with intra- and perineural monocytic and macrophagic invasion and increased levels of oxidative stress by-products (hydrogen peroxide and 4-hydroxynonenal). Attenuation of monocyte/macrophage increase by systemic treatment with an antibody against the monocyte chemoattractant chemokine (C-C motif) ligand 2 (CCL2) or the macrophage-depleting agent, clodronate (both $P < 0.05$), was associated with reduced hydrogen peroxide and 4-hydroxynonenal perineural levels and pain-like behaviours (all $P < 0.01$), which were abated by perineural administration of HC-030031, α -lipoic acid or the anti-CCL2 antibody (all $P < 0.001$). The present findings propose that, in the constriction of the infraorbital nerve model of trigeminal neuropathic pain, pain-like behaviours are entirely mediated by the TRPA1 channel, targeted by increased oxidative stress by-products released from monocytes and macrophages clumping at the site of nerve injury.

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Abbreviations: 4-HNE = 4-hydroxynonenal; AITC = allyl isothiocyanate; A-967079 = (1*E*,3*E*)-1-(4-Fluorophenyl)-2-methyl-1-penten-3-one oxime; CION = constriction of the infraorbital nerve; HC-030031 = (2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-*N*-(4-isopropylphenyl) acetamide); LCL = liposome-encapsulated clodronate

Introduction

Trigeminal neuropathic pain arises in a variety of orofacial painful conditions (Zakrzewska, 2013), which include typical (type 1) trigeminal neuralgia, characterized by excruciating and sudden pain generated by subthreshold mechanical stimuli or cold exposure, and atypical (type 2) trigeminal neuralgia, associated with background pain of lower intensity between sharp painful poussées (Zakrzewska and Linskey, 2014). Insult of the nerve trunk caused by degeneration or mechanical compression produced by various aetiologies (immunological, metabolic, viral, vascular, cancerous, traumatic or surgical) is the most plausible cause of trigeminal neuropathic pain (Zakrzewska, 2013). Patient treatment is far from satisfactory for many reasons, including uncertainty regarding the underlying mechanisms that cause trigeminal neuropathic pain from the original injury (Zakrzewska, 2013; Zhang *et al.*, 2013; Zakrzewska and Linskey, 2014).

Emerging evidence points to the transient receptor potential ankyrin 1 (TRPA1) channel as a major pain transducer (Andrade *et al.*, 2012; Nassini *et al.*, 2014). TRPA1, co-expressed with the transient receptor potential vanilloid 1 (TRPV1) channel by a subpopulation of peptidergic somatosensory neurons, is activated by plant-derived compounds, such as cinnamaldehyde, allyl isothiocyanate (AITC) and allicin (Nilius *et al.*, 2007). Additionally, an unprecedented series of reactive oxygen, nitrogen or carbonyl species, including hydrogen peroxide, peroxyxynitrite, 4-hydroxynonenal (4-HNE) and acrolein, have been identified as selective TRPA1 agonists (Bautista *et al.*, 2006; Trevisani *et al.*, 2007; Andersson *et al.*, 2008; Sawada *et al.*, 2008; Taylor-Clark *et al.*, 2009). Channel silencing and pharmacological antagonism reduced chemical, thermal (cold) and mechanical hypersensitivity in different models of neuropathic pain in peripheral nerves, including streptozotocin-induced diabetic neuropathy (Wei *et al.*, 2009), chemotherapeutic peripheral neuropathy (Nassini *et al.*, 2011; Materazzi *et al.*, 2012; Trevisan *et al.*, 2013) and sciatic nerve ligation (Obata *et al.*, 2005; Katsura *et al.*, 2006; Caspani *et al.*, 2009). However, there is no information regarding the role of TRPA1 in the various pain-like behaviours produced by nerve injury in models of trigeminal neuropathic pain.

The aim of the present study was to identify the role of TRPA1 in a mouse model of trigeminal neuropathic pain produced by the constriction of the infraorbital nerve (Luiz *et al.*, 2010) and to explore the molecular and cellular pathways that, from the initial nerve injury, result in channel engagement. The monocyte chemoattractant protein 1 (MCP-1), also known as chemoattractant chemokine (C-C

motif) ligand 2 (CCL2), by binding to the chemotactic cytokine receptor 2 (CCR2), promotes monocyte transendothelial migration to the site of nerve injury (Siebert *et al.*, 2000). In various paradigms of peripheral nerve injury, CCL2 inhibition and CCR2 genetic ablation abrogate mechanical allodynia (Abbadie *et al.*, 2003; Melgarejo *et al.*, 2009). In addition, antioxidants have been reported to attenuate neural hypersensitivity in various models of neuropathic pain, such as sciatic chronic constriction injury (Khalil *et al.*, 1999) and spinal nerve ligation (Kim *et al.*, 2004). Thus, the contribution of monocyte/macrophage infiltration and the ensuing oxidative stress in TRPA1-mediated pain-like behaviours was investigated in the constriction of the infraorbital nerve model. Results propose that CCL2-driven monocyte/macrophage accumulation within the injured nerve and the neighbouring tissue generates oxidative burst that, by TRPA1 targeting, promotes and maintains constriction of the infraorbital nerve-evoked pain-like behaviours.

Materials and methods

Animals and drugs

In vivo experiments and tissue collection were carried out according to the European Union guidelines for animal care procedures and the Italian legislation (DLgs 26/2014) application of the EU Directive 2010/63/EU. Studies were conducted under the University of Florence research permit #204/2012-B. C57BL/6 mice (male, 20–25 g, age 5 weeks, Harlan Laboratories), littermate wild-type (*Trpa1*^{+/+}) and TRPA1-deficient (*Trpa1*^{-/-}) mice (25–30 g, age 5–8 weeks), generated by heterozygotes on a C57BL/6 background (B6; 129P-Trpa1tm1Kykw/J; Jackson Laboratories; Kwan *et al.*, 2006) were used. Animals were housed in a temperature- and humidity-controlled vivarium (12 h dark/light cycle, free access to food and water, 10 animals per cage). Behavioural experiments were performed after 1 h of animal acclimation in a quiet, temperature-controlled room (20–22 °C) between 9 a.m. and 5 p.m. with a randomized order by an operator blinded to genotype and drug treatments. Animals were euthanized with a high dose of sodium pentobarbital (200 mg/kg intraperitoneally). HC-030031 [2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-*N*-(4-isopropylphenyl) acetamide] was synthesized as previously described (Andre *et al.*, 2008). If not otherwise indicated, reagents, including A-967079 [(1*E*,3*E*)-1-(4-fluorophenyl)-2-methyl-1-penten-3-one oxime] were obtained from Sigma-Aldrich.

Constriction of the infraorbital nerve

Constriction of the infraorbital nerve (CION) was performed in C57BL/6, *Trpa1*^{+/+} or *Trpa1*^{-/-} mice as previously

described (Vos *et al.*, 1994; Luiz *et al.*, 2010). Briefly, mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (3 mg/kg) and an incision was made in the left upper lip skin lateral to the nose, and the rostral end of the infraorbital nerve was exposed. Then, two loosely constrictive ligatures (#6/0 silk suture) were placed around the infraorbital nerve with a distance of 2 mm. In the sham procedure, the left infraorbital nerve was exposed but not ligated. To verify whether an inflammatory component, due to a foreign body, contributes to immune cell accumulation and mechanical and cold hypersensitivity, a silk thread was inserted close to the infraorbital nerve without any ligature. Neomycin sulphate and sulfathiazole (powder, 0.05 and 9.95 g, respectively; Boehringer Ingelheim) were applied to the wound and the incision was sutured. Mice were monitored, adequately rehydrated, and maintained in a controlled temperature (37°C) until fully recovered from anaesthesia.

Experimental design

C57BL/6 ($n = 370$) and *Trpa1*^{+/+} ($n = 48$) or *Trpa1*^{-/-} ($n = 48$) mice were randomly allocated for CION or sham surgery. Ten days after surgery, some C57BL/6 mice ($n = 128$) were randomly allocated to treatment with intragastric ($n = 8$), intraperitoneal ($n = 8$) or subcutaneous ($n = 16$), into the left upper lip, ipsilateral to the surgery ($n = 8$), or the right upper lip, contralateral to the surgery ($n = 8$), administration of the TRPA1 selective antagonists, HC-030031 (300 mg/kg or 100 µg/10 µl/site, respectively, $n = 24$), or A-967079 (100 mg/kg, intraperitoneal, $n = 8$) or the antioxidant compound, α -lipoic acid (100 mg/kg or 10 µg/10 µl/site, $n = 24$), or apocynin [(inhibitor of NADPH oxidase, NOX) 100 mg/kg or 1 µg/10 µl/site, $n = 24$], or indomethacin [30 mg/kg, intraperitoneally $n = 8$, intragastrically $n = 8$ or subcutaneously $n = 16$ (eight for each lip side)] or intraperitoneal ($n = 8$) vehicles (1% of dimethyl sulphoxide in isotonic saline, NaCl 0.9%, respectively). Nociceptive responses were assessed 0.5, 1, 2 and 3 h after drug administration. Doses and schedules of drug administration were based on previous data (McNamara *et al.*, 2007; Eid *et al.*, 2008; Trevisan *et al.*, 2013). In another group of C57BL/6 mice ($n = 16$), HC-030031 (300 mg/kg, intragastric $n = 8$) or its vehicle ($n = 16$) were administered 30 min before and (four times after at 90 min intervals) after CION or sham procedures.

To deplete the monocyte/macrophage population transiently (Old *et al.*, 2014), a different group of C57BL/6 mice, including CION- ($n = 64$) and sham- ($n = 48$) operated animals, were treated either systemically (40 µg/200 µl, intraperitoneal: CION $n = 8$, sham $n = 8$) or locally (4 µg/10 µl/site, subcutaneous: CION $n = 16$, sham $n = 8$) with an antibody directed to the CCL2 chemokine (R&D System) or its vehicle (IgG2B Isotype Control, R&D System; one injection/day starting from Day 8 after surgery until Day 10, intraperitoneally or subcutaneously into the left upper lip, ipsilateral to the surgery, or the right upper lip, contralateral to the surgery) for the two routes of administration (intraperitoneal: CION $n = 8$, sham $n = 8$; subcutaneous: CION $n = 16$, sham $n = 8$). Two additional groups of C57BL/6 mice randomly received liposome-encapsulated clodronate [LCL (ClodronateLiposomes); 5 mg/ml, intraperitoneal: CION $n = 8$, sham $n = 8$], or its vehicle [liposome-encapsulated

phosphate buffer saline (ClodronateLiposomes); CION $n = 8$, sham $n = 8$] at Day 7 and 10 after surgery (Fig. 1A).

Assessment of pain-like behaviours

In C57BL/6 and *Trpa1*^{+/+} or *Trpa1*^{-/-} mice, non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity were assessed before surgery (baseline) and 3, 7, 10, 15 and 20 days after surgery. In C57BL/6 mice treated with HC-030031, α -lipoic acid, apocynin or their vehicles and in *Trpa1*^{+/+} or *Trpa1*^{-/-}, non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity were assessed before surgery (baseline) and at Day 10 after surgery. In C57BL/6 mice treated with CCL2 antibody or LCL, pain-like behaviours were assessed before surgery and at Day 7 (LCL) or Day 8 (CCL2 antibody) and Day 10 after surgery. In C57BL/6 mice, chemical hyperalgesia was measured before surgery and at Day 10 after surgery.

Non-evoked nociceptive behaviour

As previously reported, constriction of the infraorbital nerve induces non-evoked, continuous or recurring pain in the cutaneous region innervated by the damaged nerve—a behavioural response indicative of neuropathic pain (Vos *et al.*, 1994; Xu *et al.*, 2008). To assess changes in spontaneous facial rubbing, C57BL/6, *Trpa1*^{+/+} or *Trpa1*^{-/-} mice were placed individually in clear plexiglass boxes (7 × 9 × 11 cm) on elevated wire mesh platforms (Xu *et al.*, 2008). After 1 h of adaptation, the time spent rubbing (time that forelimbs touched ears or facial region) was recorded for 30 min.

Mechanical allodynia

The mechanical threshold was measured in C57BL/6, *Trpa1*^{+/+} or *Trpa1*^{-/-} mice using the up-and-down paradigm (Chaplan *et al.*, 1994). Animals were placed individually in a restrained apparatus designed for the evaluation of mechanical thresholds (Krzyzanowska *et al.*, 2011). Mice were habituated to room temperature for at least 1 h before the test. Then, a series of seven Von Frey hairs in logarithmic increments of force (0.008, 0.02, 0.04, 0.07, 0.16, 0.4 and 0.6 g) was used to stimulate the infraorbital nerve region, i.e. near the centre of the vibrissal pad on the hairy skin of the left upper lip (ipsilateral to the surgery side). The response was considered positive when the mouse strongly withdrew its head. The stimulation initiated with the 0.16 g filament. The von Frey hairs were applied with sufficient force to cause slight buckling, and held for ~2–4 s. Absence of response after 5 s led to use of the filament with increased weight, whereas a positive response led to use of a weaker (lighter) filament. Six measurements were collected for each mouse or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) response was calculated from these scores (Dixon, 1980; Chaplan *et al.*, 1994). Basal values were recorded before the CION or sham procedure. Mechanical allodynia was considered as a decrease in the mechanical threshold in comparison to basal (intra-animal) or sham animal (inter-animal) values.

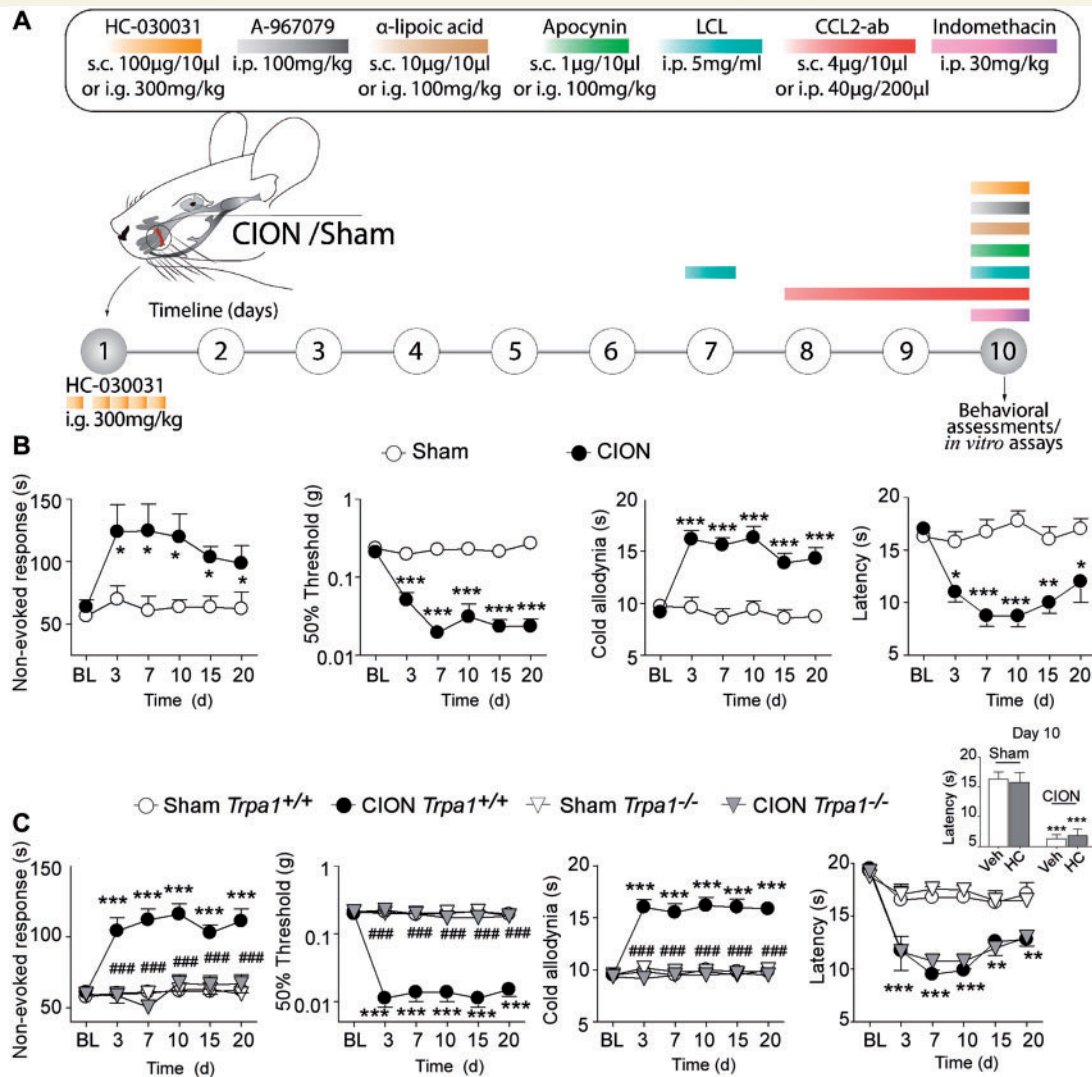


Figure 1 Constriction of the infraorbital nerve induces non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity, through TRPA1 activation in mice. (A) Scheme of the experimental procedure and timeline. C57BL/6, *Trpa1*^{+/+} or *Trpa1*^{-/-} mice underwent constriction of the infraorbital nerve or sham surgery on Day 1. At Day 10 mice received intragastric (i.g.) or subcutaneous (s.c.) HC-030031, A-967079, indomethacin, apocynin or α-lipoic acid. In another group of C57BL/6 mice, HC-030031 was administered (i.g.) 30 min before and shortly (four times at 90-min intervals) after the CION- or sham- procedures. Additional mice were treated with an antibody directed to the CCL2 chemokine (CCL2 antibody) or its vehicle (IgG2B) at Days 8, 9 and 10, or LCL or its vehicle (liposome-encapsulated PBS) at Days 7 and 10. On Day 10, in all animals, pain-like behaviours (non-evoked nociceptive behaviour, mechanical allodynia, cold hypersensitivity, chemical hyperalgesia and heat hyperalgesia) were assessed and tissues were collected for *in vitro* assays. (B) In C57BL/6 mice, constriction of the infraorbital nerve induces non-evoked nociceptive behaviour, mechanical allodynia, cold and heat hypersensitivity starting at Day 3 and persisting at Day 20 post surgery. (C) The non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity induced by CION surgery in *Trpa1*^{+/+} mice are completely absent in *Trpa1*^{-/-} mice. The heat hypersensitivity was similar in both *Trpa1*^{+/+} and *Trpa1*^{-/-} CION-operated mice. Sham-operated animals do not show any hypersensitivity when compared to basal values. *P < 0.05, **P < 0.01 and ***P < 0.001 versus sham, student's t test; ****P < 0.001 and **P < 0.01 versus sham *Trpa1*^{+/+} and sham/vehicle, one-way ANOVA and Bonferroni *post hoc* test; #####P < 0.001 versus CION *Trpa1*^{+/+}; one-way ANOVA and Bonferroni *post hoc* test. BL = baseline assessment before surgery.

Cold hypersensitivity

Cold hypersensitivity was assessed by measuring the acute nociceptive response to the acetone-evoked evaporative cooling in C57BL/6, *Trpa1*^{+/+} or *Trpa1*^{-/-} mice (Constandil *et al.*, 2012; Materazzi *et al.*, 2012). Briefly, mice were placed individually in clear plexiglass boxes (7 × 9 × 11 cm) on elevated wire mesh platforms and habituated for at least 1 h before the

test. Acetone (15 µl) was gently applied to the left vibrissal pad skin surface (ipsilateral to the surgery side), and the time spent grooming the region over a 60-s period was measured. Acetone was applied three times at 10–15 min intervals, and the average nociceptive (grooming) time was calculated. Cold allodynia was considered as an increase in the nociceptive time observed after exposure to acetone when compared with basal (intra-animal) or sham-operated animal (inter-animal) values.

Chemical hyperalgesia

Basal nociceptive behaviour was assessed by measuring spontaneous nociceptive responses induced by subcutaneous (10 μ l) injection into the left upper lip (ipsilateral to the surgery site) of increasing doses of allyl isothiocyanate (AITC, 0.1–30 nmol/site) or vehicle (dimethyl sulphoxide 3%), hydrogen peroxide (0.01–1 μ mol/site) or vehicle (isotonic saline), capsaicin (0.01–1 nmol/site) or vehicle (ethanol 1%) or concentrations of hypotonic saline (0.63%–0% NaCl/site) in non-operated C57BL/6 mice, in order to identify the minimal suprathreshold dose. Each animal was tested with one dose of each substance. The identified suprathreshold doses of AITC (1 nmol/site), hydrogen peroxide (0.1 μ mol/site), capsaicin (0.01 nmol/site) or hypotonic saline (0.45% NaCl/site) were tested in infraorbital nerve- or sham-operated mice on Day 10 after surgery. Animals were placed individually in chambers (transparent glass cylinders of 20 cm in diameter) and adapted for 20 min before algogen or vehicle injection. Each animal was tested with one suprathreshold dose of each substance or vehicle, according to a random allocation. Immediately after the injection, mice were placed inside a plexiglass box and time spent in ipsilateral facial rubbing was recorded for 5 min.

Rotarod test

Locomotor function, coordination and sedation of animals were tested by using a rotarod apparatus (UgoBasile). The test was performed as previously described (Trevisan *et al.*, 2012). Briefly, 24 h before the experiments, the animals were trained on the rotarod apparatus, programmed at 8 rpm, until they remained without falling for 60 s. The day of the experiment, the latent period (s) to the first fall and the number of falls were recorded. Cut-off time was 240 s. The results of the rotarod test (not shown) indicated that the various pharmacological interventions did not affect the forced locomotion of animals.

Thermal heat hyperalgesia

Thermal heat hyperalgesia of the orofacial area was measured in C57BL/6, *Trpa1*^{+/+} or *Trpa1*^{-/-} mice with a radiant heat (50 \pm 1 $^{\circ}$ C) placed on the surface of the vibrissal pad. The latent period before head withdrawal or vigorous flicking of the snout was recorded. A 20 s cut-off time was used to prevent tissue damage. Reductions in the response latency to heat stimulation were considered to be indicative of thermal hyperalgesia (Luiz *et al.*, 2010).

Protein extraction and western immunoblot assay

Infraorbital nerves or trigeminal ganglia were obtained from C57BL/6 mice at Day 10 after the constriction of the infraorbital nerve ($n = 12$) or sham ($n = 12$) surgery. Tissue samples were homogenized in lysis buffer containing (mM): 50 Tris, 150 NaCl, 2 EGTA, 100 NaF, 1 Na₃VO₄, 1% Nonidet P40 (pH 7.5) and complete protease inhibitor cocktail (Roche Diagnostics). Lysates were centrifuged at 14 000g at 4 $^{\circ}$ C for 45 min. Protein concentration in supernatants was determined using a DC protein assay (Bio-Rad). Samples with equal

amounts of proteins (30 μ g) were then separated by NuPAGE[®] 4–12% Bis-Tris gel electrophoresis (Life Technologies), and the resolved proteins were transferred to a polyvinylidene difluoride membrane (Merck Millipore). Membranes were incubated with 5% dry milk in Tris buffer containing 0.1% Tween 20 (TBST; 20 mM Tris at pH 7.5, 150 mM NaCl) for 1 h at room temperature, and incubated with rat polyclonal primary antibody for TRPA1 detection (1:200, Novus Biologicals), or mouse monoclonal primary antibody for β -actin (1:6000, Thermo Scientific), at 4 $^{\circ}$ C overnight. Membranes were then probed with goat anti-mouse or donkey anti-rabbit IgG conjugated with horseradish peroxidase (Bethyl Laboratories Inc.) for 50 min at room temperature. Finally, membranes were washed three times with TBST, and bound antibodies were detected using chemiluminescence reagents (ECL, Pierce, Thermo Scientific). Negative controls were obtained by overnight preadsorption at 4 $^{\circ}$ C with 1 μ g peptide/1 μ g antibody of the immunizing peptide (Novus Biological). The density of specific bands was measured using an image processing program (ImageJ 1.32J, National Institutes of Health, Bethesda, USA) and normalized to β -actin (Trevisan *et al.*, 2013).

CCL2 enzyme-linked immunosorbent assay, hydrogen peroxide level and superoxide dismutase activity

For the three different assays, left upper lips, containing the infraorbital nerve and perineural tissue, were obtained from C57BL/6 mice at Day 10 post CION ($n = 6$ for each assay) or sham ($n = 6$ for each assay) surgery. The CCL2 content in infraorbital nerve and surrounding tissue was measured by using a mouse CCL2/monocyte chemoattractant protein 1 Quantikine[®] enzyme-linked immunosorbent assay kit (R&D System). Infraorbital nerve samples were homogenized in phosphate-buffered saline (PBS) at 4 $^{\circ}$ C containing a protease inhibitor cocktail tablet (Roche Diagnostics). The homogenate was then centrifuged at 10 000g for 20 min at 4 $^{\circ}$ C, supernatants were collected and assayed according to the manufacturer's instructions. The concentration of CCL2 was expressed in pg/mg of total protein content (Bradford, 1976).

Hydrogen peroxide levels in infraorbital nerves were detected by using the phenol red-horseradish peroxidase method (Nakamura *et al.*, 1998; Trevisan *et al.*, 2013). Samples were homogenized in 50 mM phosphate buffer (pH 7.4) containing 5 mM of sodium azide at 4 $^{\circ}$ C for 60 s, centrifuged at 12 000g for 20 min at 4 $^{\circ}$ C, and the supernatant was used to determine the hydrogen peroxide content. Levels of hydrogen peroxide were expressed as μ mol on the basis of a standard curve of horseradish peroxidase-mediated oxidation of phenol red by hydrogen peroxide, corrected by protein content (mg) (Bradford, 1976).

The superoxide dismutase activity was assayed using a Nitro Blue Tetrazolium (NBT)-based assay (Abcam; Oberley and Spitz, 1984). Briefly, infraorbital nerves were homogenized in a Tris-HCl buffer (100 mM, pH 7.4) containing 0.5% Triton[™] X-100, 5 mM beta-mercaptoethanol, 0.1 g/ml phenylmethanesulfonyl fluoride, centrifuged at 14 000g at 4 $^{\circ}$ C for 5 min and assayed according to the manufacturer's

instructions. Results were expressed as the percent inhibition of the rate of NBT-diformazan formation.

Immunofluorescence assay

For biochemical or histological analyses the ligature region and the respective proximal and distal areas of the infraorbital nerve were dissected and the suture silk removed before preparation of tissue homogenates or rotary microtome slicing. Tissues were obtained from C57BL/6 mice at Day 10 after CION ($n = 6$) or sham ($n = 6$) surgery. Mice were anaesthetized with a mixture of ketamine (90 mg/kg) and xylazine (3 mg/kg) and transcardially perfused with PBS, followed by 4% paraformaldehyde. The infraorbital nerve with the surrounding tissue were removed, placed in 4% paraformaldehyde, and then embedded in paraffin. Immunofluorescence staining was performed according to standard procedures. Briefly, after antigen retrieval (EDTA solution pH 9.0, Dako) for 20 min at 98 °C, sections (4 μ m) were incubated with the following primary antibodies: F4/80 (1:50, Abcam), protein gene product 9.5 (PGP9.5, 1:600, Abcam), TRPA1 (1:400, AVIVA System Biology) or 4-HNE (1:40, HNEJ-2, Abcam) diluted in fresh blocking solution (PBS, pH 7.4, 5 mg/ml bovine serum albumin and 2.5% normal goat serum) and applied 1 h at room temperature. Sections were then incubated for 2 h in the dark with a fluorescent secondary antibody (polyclonal Alexa Fluor® 488 FITC-conjugated, and polyclonal Alexa Fluor 594 TRITC-conjugated, Invitrogen) diluted 1:600 in blocking solution (PBS, pH 7.4, 5 mg/ml bovine serum albumin and 2.5% normal goat serum). Sections were coverslipped using a water-based mounting medium with 4',6'-diamidino-2-phenylindole (DAPI, Abcam). The analysis of negative controls (non-immune serum) was simultaneously performed in order to exclude the presence of non-specific immunofluorescent staining, cross-immunostaining or fluorescence bleed-through. For histological evaluation, sections were stained with haematoxylin/eosin, and based on the morphology, the boundaries of the nerve trunk corresponding to the epineurium were identified and reported in adjacent immunofluorescence images with dashed lines. The number of F4/80⁺ cells was counted in 10⁴ μ m² boxes within the dashed lines of the injured branches of the infraorbital nerve. The 4-HNE staining was evaluated as the fluorescence intensity measured by an image processing program (ImageJ 1.32J, National Institutes of Health, Bethesda, USA).

Electrophysiology

Trigeminal ganglion neurons were isolated from C57BL/6 mice at Day 10 after CION ($n = 6$) and sham ($n = 6$) surgery, and whole-cell patch-clamp recordings were performed 24 h after cell isolation (Nassini *et al.*, 2012; Fusi *et al.*, 2014). Trigeminal ganglion neurons isolated from CION- and sham-operated mice were perfused with AITC (30 μ M) and capsaicin (1 μ M). Peak currents activated by each compound were normalized to cell membrane capacitance and expressed as mean of the current density (pA/pF) in averaged results. Currents were evoked in the voltage-clamp mode at a holding potential of -60 mV; signals were sampled at 1 kHz and low-pass filtered at 10 kHz.

Statistical analysis

Data are presented as mean \pm SEM. Statistical analysis was performed by the unpaired two-tailed Student's *t*-test for comparisons between two groups, the one- or two-way ANOVA, followed by the *post hoc* Bonferroni's test for comparisons of multiple groups. $P < 0.05$ was considered statistically significant (GraphPad Prism version 5.00). To meet ANOVA assumptions, mechanical allodynia data were subjected to log transformation before statistical analysis.

Results

Constriction of the infraorbital nerve induces pain-like behaviors via TRPA1 activation

Constriction of the infraorbital nerve induced significant changes in non-evoked nociceptive response and mechanical allodynia in C57BL/6 mice at Day 3 after surgery and throughout the 20 days of observation, whereas in sham-operated mice the three outcomes remained stable over the entire period of observation (Fig. 1B). Constriction of the infraorbital nerve also induced hypersensitivity to cold (Fig. 1B). Infraorbital nerve and sham operation did not affect normal body weight increase (not shown). As previously reported (Luiz *et al.*, 2010), in C57BL/6 mice, constriction of the infraorbital nerve decreased the response latency to the application of the heat stimulus compared to the sham-operated group (Fig. 1B). Treatment with HC-030031 did not affect heat hyperalgesia at Day 10 after surgery (Fig. 1C). In addition, heat hyperalgesia produced by constriction of the infraorbital nerve was similar in both *Trpa1*^{+/+} and *Trpa1*^{-/-} mice (Fig. 1C). As constriction of the infraorbital nerve-evoked heat hyperalgesia is independent from TRPA1, and heat does not seem to play a major role as a trigger or an aggravating factor in trigeminal neuropathic pain (Eide and Rabben, 1998; Zakrzewska, 2013), heat hyperalgesia was not further investigated.

In wild-type (*Trpa1*^{+/+}) mice, changes in non-evoked nociceptive behaviour and mechanical allodynia or cold hypersensitivity produced by constriction of the infraorbital nerve were similar to those observed in C57BL/6 mice (Fig. 1C). In contrast, and most importantly, littermate *Trpa1*^{-/-} mice were completely protected from all pain-like behaviours evoked by constriction of the infraorbital nerve (Fig. 1C). At Day 10 post surgery, systemic (intragastric) treatment with the TRPA1 selective antagonist, HC-030031, completely reverted the non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity (Fig. 2A). HC-030031 did not affect baseline values in sham-operated animals (Fig. 2A). The same results were obtained with another TRPA1 selective antagonist, A-967079 (Chen *et al.*, 2011). Systemic (intraperitoneal) A-967079 completely reverted non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity

evoked by constriction of the infraorbital nerve (Fig. 2B). As for HC-030031, A-967079 did not affect baseline values in sham-operated animals (Fig. 2B). In addition, to assess whether TRPA1 inhibition prevents the development of non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity, HC-030031 was administered just before and four times (every 90 min) after the CION or sham procedures. Such treatment delayed the onset of pain-like behaviours by ~15 days (Fig. 2C), which fully recurred 15–20 days after constriction of the infraorbital nerve.

To identify the site of TRPA1 engagement, HC-030031 was administered locally. At Day 10 after surgery, local (subcutaneous) injection of HC-030031 in the left upper lip, ipsilateral to the surgery, reverted the non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity (Fig. 2D). Importantly, HC-030031 injection in the right upper lip, contralateral to the surgery side, did not affect any pain-like behaviours measured in the ipsilateral upper lip (Fig. 2D). HC-030031 (subcutaneous) did not change any pain-like parameters in sham-operated mice (Fig. 2D). However, due to the vicinity of the injection site to both the ligature site and the skin area where Von Frey hairs are applied, it is possible that HC-030031 diffuses to both of them. Accordingly, these experiments cannot distinguish if only one or both of the two areas along the nerve fibre are targeted by the channel antagonist.

There is evidence that nerve injury associated with surgical procedures affects channel expression in different sections of sensory nerves (Gillen *et al.*, 1995; Li *et al.*, 2013; Jiang *et al.*, 2014). We evaluated, by western blotting, TRPA1 protein content in the infraorbital nerve both ipsilateral and contralateral to the surgery in either constriction of the infraorbital nerve- or sham-operated mice. Two major bands were identified, one slightly above 100 kDa and the other slightly below 140 kDa. In the presence of the immunizing peptide, the 100 kDa band disappeared, thus indicating this band as the one most likely to correspond to the TRPA1 (Fig. 3A). At Day 10 after surgery, TRPA1 protein expression was not changed in infraorbital nerve (Fig. 3A) across the four different experimental conditions.

Selective chemical hypersensitivity to TRPA1 agonists has been reported in experimental neuropathic pain (Trevisan *et al.*, 2013). Local injection (subcutaneous) in the left upper lip of the TRPA1 agonists, AITC or hydrogen peroxide, the transient receptor potential vanilloid 1 (TRPV1) selective agonist, capsaicin and hypotonic saline, which stimulates the TRPV4 channel (Alessandri-Haber *et al.*, 2003; Trevisan *et al.*, 2013), evoked a dose-dependent increase in the nociceptive behaviour in naïve, non-operated C57BL/6 mice (not shown). The nociceptive responses produced by suprathreshold doses of AITC and hydrogen peroxide, but not those evoked by capsaicin or hypotonic saline, were more intense in CION-operated than in sham-operated mice (Fig. 3B–E). Notably, *Trpa1*^{-/-} mice showed neither acute nociception in response to suprathreshold doses of AITC and hydrogen peroxide, nor increased

responses to these stimuli after constriction of the infraorbital nerve (Fig. 3F and G). However, in CION-operated *Trpa1*^{+/+} mice the nociceptive responses produced by suprathreshold doses of AITC and hydrogen peroxide were similar to those observed in C57BL/6 mice (Fig. 3F and G).

The primary role of TRPA1 in CION-evoked hypersensitivity is further supported by *in vitro* electrophysiological experiments performed in cultured trigeminal ganglion neurons obtained 10 days after the CION or sham procedures. Inward currents produced in neurons from CION-operated mice by a suprathreshold concentration of AITC were higher than those obtained in neurons from sham-operated mice (Fig. 3H). In contrast, the response to capsaicin was similar in neurons from CION- or sham-operated mice (Fig. 3H). In spite of the exaggerated functional response, TRPA1 protein expression was unchanged in trigeminal ganglia of CION or sham mice (Fig. 3I). Thus, TRPA1 hypersensitivity in constriction of the infraorbital nerve does not seem to depend on increased protein expression.

Oxidative stress mediates pain-like behaviors induced by constriction of the infraorbital nerve

At Day 10 after constriction of the infraorbital nerve, changes in non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity were abrogated 1 h after the systemic (intra-gastric) administration of the antioxidant agent, α -lipoic acid (Fig. 4A). A similar complete attenuation was obtained after local treatment (subcutaneous) with α -lipoic acid into the left upper lip, ipsilateral to the surgery (Fig. 4B). Instead, local administration of α -lipoic acid to the contralateral side did not afford any protection against pain-like behaviours (Fig. 4B). In addition, at Day 10 after constriction of the infraorbital nerve surgery and 1 h after intra-gastric or subcutaneous (left upper lip, ipsilateral to the surgery side) administration of the non-selective NOX inhibitor, apocynin, abated non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity (Fig. 4C and D). Thresholds of sham-operated mice were not affected by either α -lipoic acid or apocynin, independently from their route of administration (Fig. 4A–D).

Constriction of the infraorbital nerve induces local monocyte/macrophage infiltration

At Day 10 after surgery, a number of infiltrating monocytes/macrophages were observed in the infraorbital nerve of CION-operated mice. At Day 10 after sham operation or sham operation with the insertion of the silk thread without ligature, only a few macrophages were found in infraorbital nerve (Fig. 5A and B). In addition, CCL2 levels in infraorbital nerve tissue homogenates from CION-operated mice were markedly augmented (Fig. 5C). In addition,

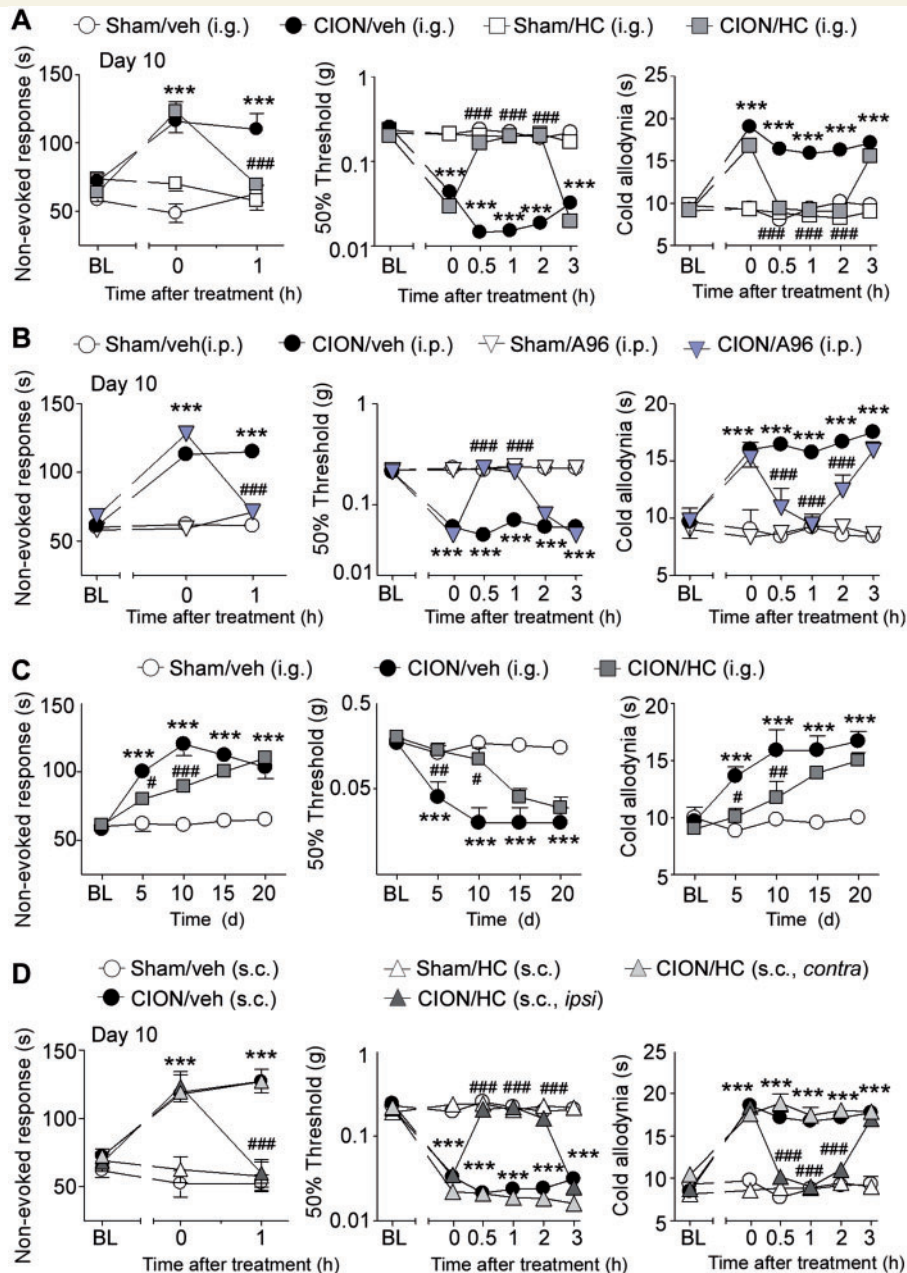


Figure 2 Pharmacological inhibition of TRPA1 prevents the non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity induced by constriction of the infraorbital nerve. (A and B) At Day 10 after surgery, the systemic administration of selective TRPA1 receptor antagonists, HC-030031 (HC, 300 mg/kg intragastric, i.g.) or A-967079 (A96, 100 mg/kg intraperitoneal, i.p.), transiently reverses non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity 1 h post dosing. (C) HC-030031 (300 mg/kg, i.g.) administered just before and four times at 90 min intervals after CION or sham procedures prevents the early, but not the late phase of non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity induced by constriction of the intraorbital nerve. (D) At Day 10 after CION surgery, the subcutaneous (s.c.) administration of HC-030031 (100 µg/10 µl) in the left upper lip, ipsilateral (ipsi) to CION surgery, but not its injection in the contralateral (contra) upper lip, completely reverses the non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity. (A–D) HC-030031 (s.c. and i.g.) and A-967079 (i.p.) do not affect the non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity 30 min, 1 and 2 h post dosing at Day 10 after sham procedure. *** $P < 0.001$ versus sham/vehicle, sham/HC and sham/A96, one-way ANOVA and Bonferroni *post hoc* test; # $P < 0.05$, ### $P < 0.01$ and #### $P < 0.001$ versus CION/vehicle; one-way ANOVA and Bonferroni *post hoc* test. BL = baseline assessment before surgery.

CCL2 levels in homogenates of the nerve trunk and the surrounding tissue were augmented in CION-operated mice (Fig. 5C). A reduction in monocyte/macrophage content was observed in CION-operated mice treated

systemically (intraperitoneally) with the macrophage-depleting agent, LCL, which, as expected, did not affect CCL2 levels (Fig. 5B and C). In addition, the number of infiltrating monocytes or macrophages and CCL2 tissue levels were

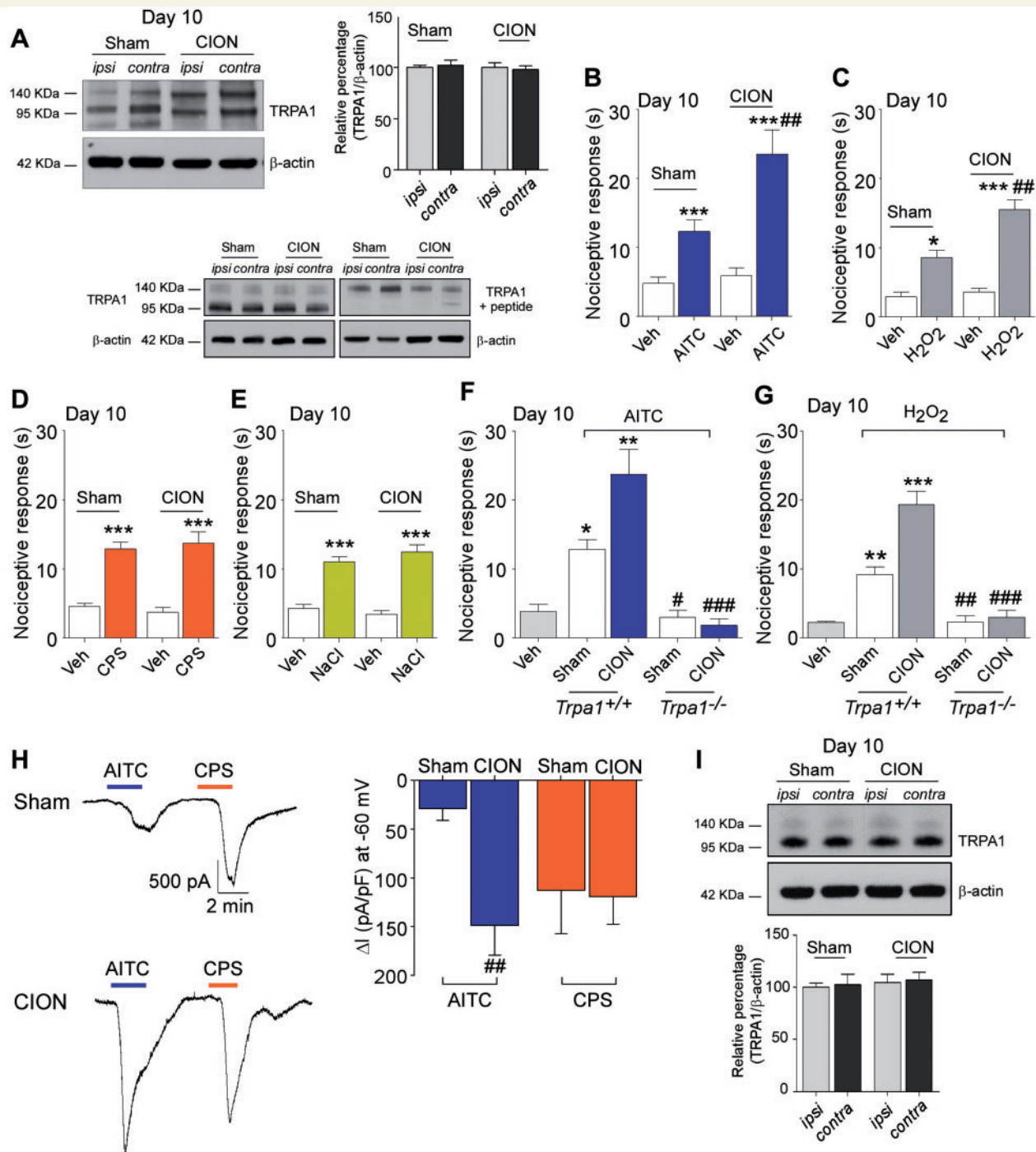


Figure 3 Constriction of the infraorbital nerve does not increase TRPA1 expression but enhances its activity. (A) TRPA1 protein content analysed by western blotting is not different in infraorbital nerve tissue homogenates obtained from the side ipsilateral (ipsi) and contralateral (contra) to the surgery in sham and CION mice 10 days after surgery. Equally loaded protein was checked by expression of β -actin. Representative blots show TRPA1 protein expression in the infraorbital nerve and negative control obtained by preadsorption with the immunizing peptide. (B and C) The nociceptive response induced by a suprathreshold subcutaneous (s.c., 10 μ l) dose of the TRPA1 agonists, AITC (1 nmol/site) or hydrogen peroxide (0.1 μ mol/site) injected in the left upper lip, ipsilateral to CION surgery, is enhanced in CION mice compared to sham mice 10 days after surgery. (D and E) The responses to suprathreshold doses of capsaicin (CPS, 0.01 nmol/site) or hypotonic saline (NaCl, 0.45%/site) are not changed in CION mice. (F and G) AITC (1 nmol/site) or hydrogen peroxide (0.1 μ mol/site) injection induces nociceptive behaviours that are increased in *Trpa1*^{+/+} CION versus sham mice. Both the nociceptive behaviour and its potentiation in CION mice are completely absent in *Trpa1*^{-/-} mice. *Trpa1*^{-/-} mice do not show any nociceptive behaviour, including CION potentiation of nociceptive response, when the two TRPA1 agonists are administered. (H) A low concentration of AITC (30 μ M) elicits an inward current in trigeminal neurons isolated from sham mice, a response that results potentiated in neurons taken from CION mice at Day 10 after surgery. (I) TRPA1 protein content analysed by western blotting is not different in trigeminal ganglion homogenates obtained from the side ipsilateral (ipsi) and

(continued)

significantly reduced by the systemic (intraperitoneal) administration at Days 8 and 10 of the CCL2 antibody as compared to the administration of the inactive IgG2B isotype (Fig. 5B and C). Finally, the failure of indomethacin to affect pain-like behaviours evoked by constriction of the infraorbital nerve indicates that infiltrating monocytes or macrophages do not promote pain-like behaviours due to a cyclooxygenase-dependent inflammatory response (Fig. 5D).

Monocytes/macrophages increase oxidative stress in the infraorbital nerve and pain-like behaviors

At Day 10 after surgery, superoxide dismutase activity and hydrogen peroxide levels were increased in the peripheral infraorbital nerve and perineural tissue homogenates from CION-operated mice as compared to sham-operated mice (Fig. 6A and B). Treatment with systemic (intraperitoneal) LCL or CCL2 antibody significantly reduced superoxide dismutase activity and hydrogen peroxide levels in CION-operated mice (Fig. 6A and B), without affecting baseline levels of sham-operated mice. To identify the site of origin of the oxidative burst, associated with the TRPA1-dependent pain-like behaviours, we measured the content of 4-HNE, a final product of peroxidation of plasma membrane phospholipids (Csala *et al.*, 2015). Staining with 4-HNE was markedly increased within the ligated infraorbital nerve and in the surrounding tissue of CION-operated as compared to sham-operated mice (Fig. 6E). Importantly, 4-HNE accumulated within or in the vicinity of TRPA1-expressing nerve bundles (Fig. 6E). TRPA1 staining was found within nerve bundles (PGP9.5 positive) of the infraorbital nerve and in some cells surrounding the nerve trunk in slices from *Trpa1*^{+/+} mice but not from *Trpa1*^{-/-} mice (Fig. 6C). The ability of the antibody to label TRPA1 was further proved by the intense staining observed in trigeminal ganglion from *Trpa1*^{+/+} mice and the absence of staining in trigeminal ganglion from *Trpa1*^{-/-} mice (Fig. 6D). The increased 4-HNE content associated with constriction of the infraorbital nerve was attenuated by systemic (intraperitoneal) administration of either the CCL2 antibody or LCL (Fig. 6E).

Importantly, monocyte/macrophage reduction by LCL or CCL2 antibody was associated with a remarkable inhibition of non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity (Fig. 7A and B). To determine whether pain-like behaviours were dependent

from monocytes/macrophages accumulated at the site of nerve injury, we administered the CCL2 antibody locally. Injection (subcutaneous) of CCL2 antibody in the left upper lip, ipsilateral to the surgery, reverted the non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity (Fig. 7C). In contrast, when the CCL2 antibody was injected in the right upper lip, contralateral to the surgery side, no change in pain-like behaviours was found in the ipsilateral left upper lip (Fig. 7C). These data indicate that the invasion of the infraorbital nerve and perineural tissue by monocytes/macrophages is a necessary and sufficient condition for the development of pain-like behaviours produced by constriction of the infraorbital nerve.

Discussion

The present findings show for the first time that TRPA1 is essential in generating pain-like behaviours in a model of mechanical injury of the trigeminal nerve, as genetic ablation of this channel totally prevented non-evoked nociceptive behaviour, mechanical allodynia and cold and chemical hypersensitivity produced by constriction of the infraorbital nerve. Remarkably, TRPA1-deleted mice were fully protected from all CION-evoked pain-like behaviours over the entire period of observation (20 days). Although spontaneous pain and mechanical allodynia are major features in the different clinical presentations of trigeminal neuropathic pain, non-noxious thermal stimuli are reported as triggers and/or worsening factors (Zakrzewska, 2013). As the effect of *TRPA1* genetic deletion in preventing, and TRPA1 pharmacological blockade in reverting, cold hypersensitivity parallels the results obtained with mechanical hypersensitivity, it may be concluded that channel engagement mediates responses in mice that recapitulate the major symptoms observed in trigeminal neuropathic pain.

The key role of TRPA1 in the maintenance of nociception evoked by constriction of the infraorbital nerve and mechanical or cold hypersensitivity is further corroborated by pharmacological findings. At Day 10, when nociception and hypersensitivity robustly persisted, systemic administration of the selective TRPA1 antagonists HC-030031 and A-967079 completely reverted all pain-like behaviours, indicating that some hitherto undefined endogenous mechanisms promote the ongoing channel activation that maintains the altered condition. Furthermore, repeated systemic treatment with HC-030031 at the time of nerve injury delayed, but did not prevent, the onset of pain-like

Figure 3 Continued

contralateral (contra) to the surgery in sham and CION mice 10 days after the surgery. Equally loaded protein was checked by expression of β -actin. Representative blot are shown. Values are mean \pm SEM of six to eight mice. * $P < 0.05$ versus sham/vehicle or vehicle or sham *Trpa1*^{+/+}, ** $P < 0.01$ versus CION/vehicle or sham *Trpa1*^{+/+}, *** $P < 0.001$ versus sham/vehicle or sham *Trpa1*^{+/+}, # $P < 0.05$ versus sham *Trpa1*^{+/+}, ### $P < 0.05$ versus sham AITC or sham hydrogen peroxide or sham *Trpa1*^{+/+}, #### $P < 0.001$ versus CION *Trpa1*^{+/+}; one-way ANOVA and Bonferroni *post hoc* test.

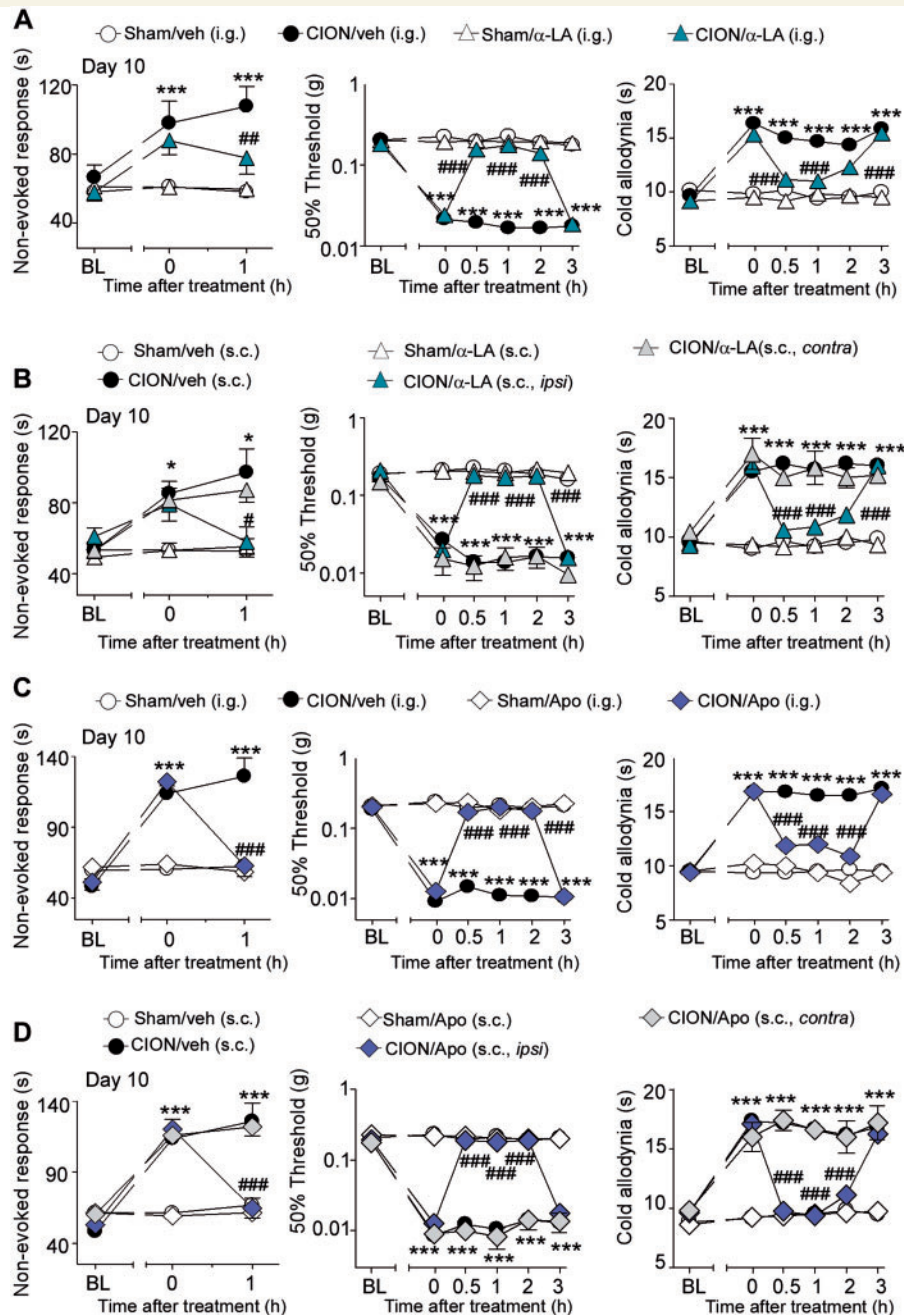


Figure 4 Systemic and local administration of α -lipoic acid or NOX inhibitor apocynin transiently reverts non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity induced by constriction of the infraorbital nerve. **(A and B)** In C57BL/6 mice, 10 days after CION surgery, both intragastric (i.g.) α -lipoic acid (α -LA) (100 mg/kg) and subcutaneous (s.c.) administration in the left upper lip, ipsilateral (ipsi) to CION surgery, but not in the contralateral (contra) upper lip, of α -LA (100 μ g/site) transiently (for 2 h starting from 30 min post dosing) abates non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity. **(C)** At Day 10 after CION surgery, apocynin [Apo; intragastric (i.g.), 100 mg/kg], 1 h after its injection, transiently reverts non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity. **(D)** A similar complete reduction in non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity is observed 1 h after s.c. administration in the left upper lip, ipsilateral (ipsi) to CION surgery, but not in the contralateral (contra) upper lip, of Apo (s.c., 1 μ g/site). Either i.g. or s.c. injection of α -LA or Apo does not affect any nociceptive behaviour evaluated in sham mice. Values are mean \pm SEM of six to eight mice * P < 0.05 and *** P < 0.001 versus sham/vehicle. # P < 0.05, ### P < 0.01 and ### P < 0.001 versus CION/vehicle; one-way ANOVA and Bonferroni *post hoc* test. BL = baseline assessment, before surgery.

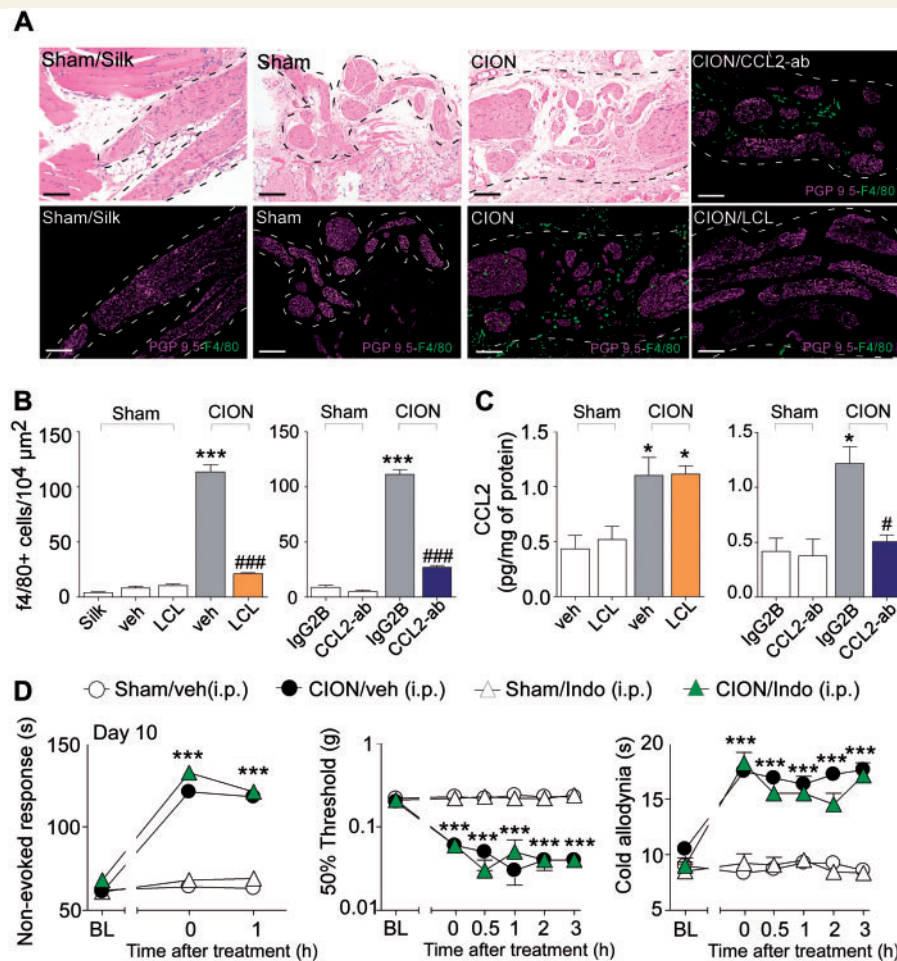


Figure 5 Constriction of the infraorbital nerve induces monocyte/macrophage infiltration in the site of ligature, which is reduced by macrophage depletion. (A and B) Representative images and pooled data of monocytes/macrophages infiltrating the nerve trunk in CION, sham or sham/silk (insertion of the silk thread without ligature) mice. Dashed lines represent the epineurium border of one of the injured branches of the infraorbital nerve. (B) Systemic intraperitoneal (i.p.) liposome-encapsulated clodronate (LCL, 5 mg/ml, injected at Days 7 and 10 after surgery) or an antibody directed to CCL2 chemokine (CCL2 antibody, 40 μg/200 μl, injected from Days 8 to 10 after surgery) prevent macrophage infiltration. (C) Pooled data of the increase in CCL2 content in tissue (infraorbital nerve and surrounding tissue) taken from the ipsilateral side to the surgery in CION mice on Day 10 after surgical procedure compared to sham. CCL2 antibody, but not LCL, prevents the increase in CCL2 content. (D) At Day 10 after surgery, i.p. administration of indomethacin (Indo, 30 mg/kg) does not affect non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity induced by constriction of the orbitofrontal nerve. Values are mean ± SEM of six to eight mice. **P* < 0.05 and ****P* < 0.001 versus sham/vehicle or sham/silk or sham/IgG2B, #*P* < 0.05 versus CION/IgG2B, ####*P* < 0.001 versus CION/vehicle or CION/IgG2B; one-way ANOVA and Bonferroni *post hoc* test. The number of F4/80⁺ cells was counted in 10⁴ μm² boxes in left upper lip sections.

behaviours, suggesting that a possible therapy with channel antagonists must consider a chronic schedule of treatment. The observation that local HC-030031 attenuated pain-like behaviours only when injected ipsilaterally to the injury indicates that TRPA1 targeting is confined to the damaged nerve. However, due to the close proximity of the injection site to both the injured nerve trunk and the skin area where hypersensitivity is assayed, the precise site of action of the TRPA1 antagonist remains unidentified.

Although fluctuations in the expression of transient receptor potential channels have been described in some rodent models of nerve injury (Gillen *et al.*, 1995; Li *et al.*, 2013; Jiang *et al.*, 2014), no change in TRPA1 protein expression was found in the infraorbital nerve under the present experimental circumstances. However, we observed a selective hypersensitivity in behavioural responses to TRPA1 agonists in CION mice. One possible interpretation is that, rather than TRPA1 upregulation, an unidentified mechanism, which is activated within the injured nerve trunk or in neighbouring tissue, engages the channel to cause the hypersensitivity. The finding that the putative endogenous channel agonist hydrogen peroxide caused an exaggerated response in CION mice points to oxidative stress by-products as possible mediators of the perpetuation

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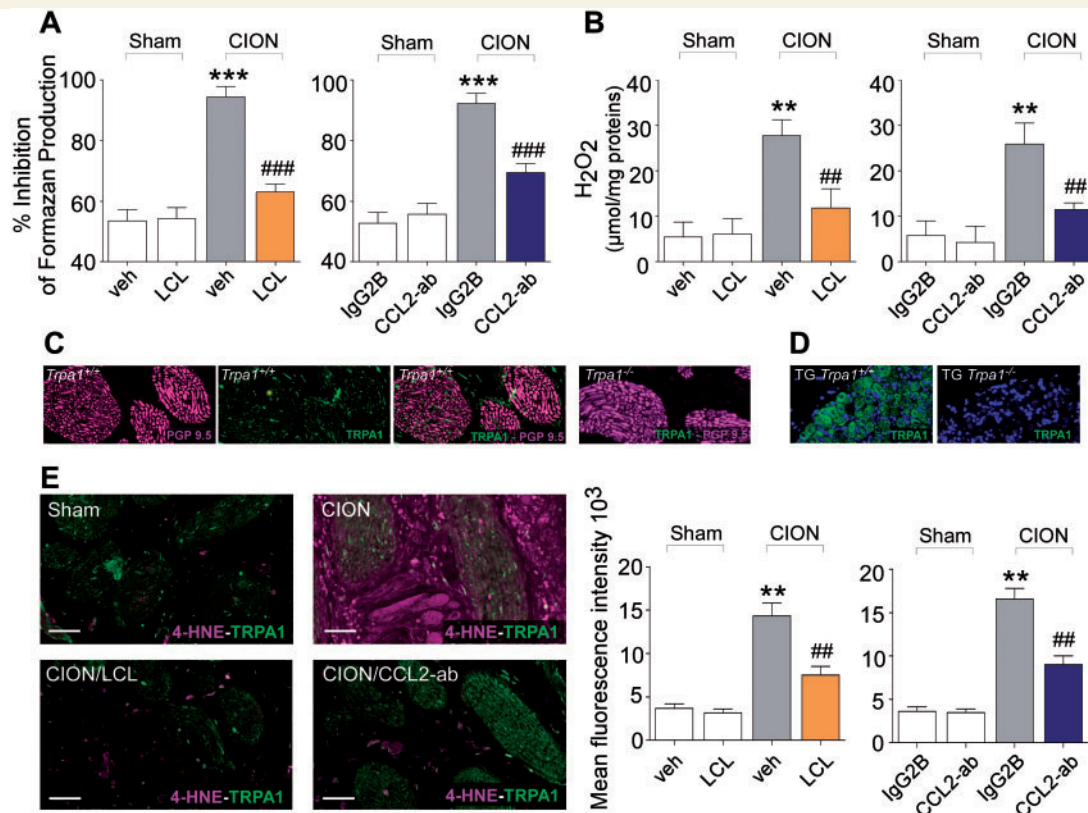


Figure 6 Constriction of the infraorbital nerve induces the increase in superoxide dismutase activity, hydrogen peroxide levels, and 4-HNE content in peripheral infraorbital nerve and perineural tissue, ipsilateral to surgery. (A and B) Superoxide dismutase activity and hydrogen peroxide content measured at Day 10 after sham or CION surgery, are reduced by intraperitoneal (i.p.) liposome-encapsulated clodronate (LCL, 5 mg/ml, injected at Days 7 and 10 after surgery) or the antibody against CCL2 (CCL2 antibody, 40 µg/200 µl, injected at Days 8 and 10 after surgery), but not by their respective vehicles. (C) TRPA1 staining is present both in PGP9.5 positive nerve bundles of infraorbital nerve and in some cells of the surrounding tissue, and in trigeminal ganglion (D) from *Trpa1*^{+/+}, but not from *Trpa1*^{-/-} mice. (E) Representative images and pooled data of the 4-HNE content. 4-HNE staining is markedly increased within the ligated infraorbital nerve and in the surrounding tissue of CION-operated mice as compared to sham-operated mice, and that increase is reverted by treatment with LCL or CCL2 antibody. Values are mean ± SEM of six mice. ***P* < 0.01 and ****P* < 0.001 versus sham/vehicle or sham/IgG2B, ###*P* < 0.01 and ####*P* < 0.001 versus CION/vehicle or CION/IgG2B; one-way ANOVA and Bonferroni *post hoc* test. BL = baseline assessment before surgery.

of TRPA1 activation. In agreement with this hypothesis, antioxidants have been used with some efficacy in a variety of neuropathic pain models, usually produced in experimental animals by injuring non-cephalic nerves (Mao *et al.*, 2009; Senoglu *et al.*, 2009; Gong *et al.*, 2012).

The observation that α -lipoic acid reverted CION-evoked spontaneous nociception and mechanical and cold hypersensitivity indicates that oxidative stress byproducts, generated by nerve injury, promote pain-like behaviours. It is well established that reactive molecules activate TRPA1, which, for this reason, is considered to be a sensor of oxidative stress (Bessac *et al.*, 2008; Nassini *et al.*, 2014). Thus, as both TRPA1 blockade and oxidative stress inhibition diminished non-evoked nociceptive behaviours and mechanical or cold hypersensitivity, it can be proposed that oxidative stress by-products mediate CION-evoked pain-like behaviours through TRPA1. As for HC-030031, ipsilateral, but not contralateral, treatment with α -lipoic acid recapitulated the protective effects obtained with

systemic antioxidant administration. Therefore, the oxidative stress byproducts needed for the TRPA1-dependent hyperalgesic phenotype must be produced in the vicinity of the injured nerve trunk. Biochemical and morphological evidence robustly supports this hypothesis.

Constriction of the infraorbital nerve increased both superoxide dismutase activity and hydrogen peroxide levels in tissue homogenates of perineural tissue, containing the injured nerve. In addition, constriction of the infraorbital nerve remarkably augmented 4-HNE staining, which was localized mainly within and around TRPA1-expressing nerve bundles. The close proximity of 4-HNE and TRPA1 channels supports the hypothesis that oxidative stress by-products are the mediators that initiate and maintain the ongoing TRPA1-dependent pain-like condition. Attenuation of nociception or hypersensitivity by apocynin, indicates NOX-dependent production of superoxide anion as the early and upstream step in the enzymatic chain that eventually results in the increased hydrogen peroxide and

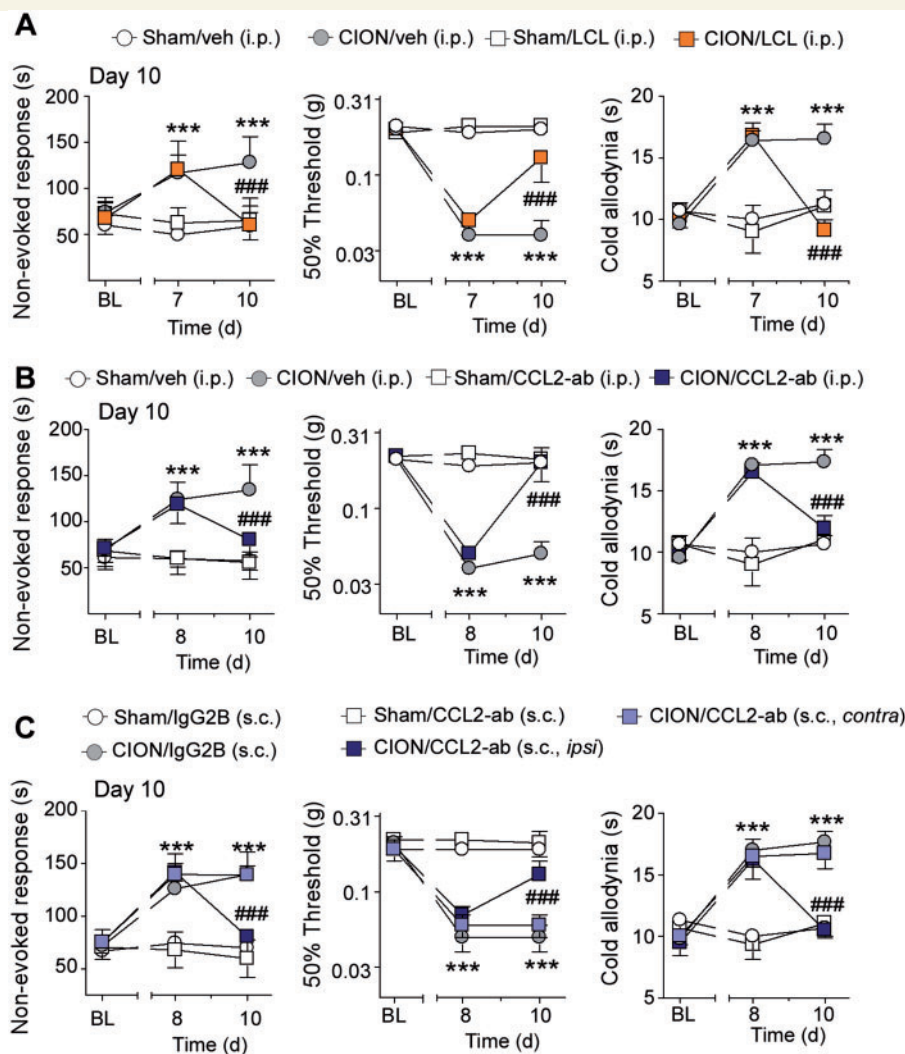


Figure 7 Systemic and local administration of LCL or CCL2 antibody reverts non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity evoked by the constriction of the infraorbital nerve. (A and B) The effect of intraperitoneal (i.p.) treatment with LCL (5 mg/ml, injected at Days 7 and 10 after surgery), the CCL2 antibody (40 µg/200 µl, injected at Days 8 and 10 after surgery) and their respective vehicles in sham and CION-operated mice. Both treatments revert non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity induced by constriction of the intraorbital nerve. (C) At Day 10 after surgery and 1 h post dosing, subcutaneous (s.c.) administration of CCL2 antibody (4 µg/10 µl, two injections starting from Day 8 after surgery) in the left upper lip, ipsilateral (ipsi) to CION surgery, but not its injection in the contralateral (contra) upper lip, completely reverts the non-evoked nociceptive behaviour, mechanical allodynia and cold hypersensitivity. Values are mean \pm SEM of six to eight mice. *** P < 0.001 versus sham/vehicle or sham/IgG2B, #### P < 0.001 versus CION/vehicle or CION/IgG2B; one-way ANOVA and Bonferroni *post hoc* test. BL = baseline assessment, before surgery.

4-HNE tissue levels and the ensuing pain-like behaviours. Reduced nociception provided by perineural application of apocynin further reinforces the proposal that oxidative stress produced locally is the main contributing factor in CION-evoked pain-like behaviours. Therefore, the most parsimonious hypothesis indicates that in the CION mouse model, increased oxidative stress byproducts are required to activate neuronal TRPA1, which promotes pain-like behaviours, and that these events are initiated and persist over time within and in the vicinity of the ligated nerve trunk. Nevertheless, present findings do not exclude that after initial events occurring at the injured

nerve trunk, upstream sites in the pain pathway exaggerate nociceptive signals. Although TRPA1 protein expression was not increased in trigeminal ganglion neurons after constriction of the infraorbital nerve, the observation that freshly dissociated trigeminal ganglion neurons are selectively hypersensitive to TRPA1 activation is in line with this hypothesis. A variety of mechanisms, independent from protein overexpression have been proposed to regulate TRPA1 functionality and sensitization. These mechanisms include increased channel translocation to the plasma membrane (Schmidt *et al.*, 2009), activation of phospholipase C (Dai *et al.*, 2007) or protein kinase A (Wang *et al.*, 2008)

and changes in intra- and extra-cellular calcium (Doerner *et al.*, 2007; Zurborg *et al.*, 2007). However, the identification of additional contributing mechanisms to the CION-evoked hypersensitivity in the trigeminal ganglion or in the CNS is beyond the purpose of this study.

Neuropathic pain following nerve injury has long been known to be associated with Wallerian degeneration, which is hallmarked by local infiltration of inflammatory cells (Ramer *et al.*, 1997; Gaudet *et al.*, 2011). However, uncertainty remains regarding the mechanisms by which pain symptoms result from such cellular recruitment and activation. In the present mouse model, as previously found in sciatic nerve injury paradigms (Komori *et al.*, 2011) and partially reported in a CION rat model (Nakai *et al.*, 2010), we found a remarkable increase in the monocytes/macrophages, which accumulated at the site of nerve damage. The ability of the monocyte/macrophage depleting agent, clodronate, to attenuate the increase in hydrogen peroxide, 4-HNE tissue levels and nociception/hypersensitivity underlines the essential role of cellular infiltration in CION-evoked pain-like behaviours. Genetic or pharmacological inhibition of the CCL2-CCR2 pathway was reported to attenuate inflammatory cell accumulation and hyperalgesia in a mouse model (sciatic nerve ligation) of neuropathic pain (Abbadie *et al.*, 2003). Previous reports (Hackel *et al.*, 2013; Pflucke *et al.*, 2013) showed that, in rats, CCL2 intraplantar injection increased monocytes/macrophages and 4-HNE and produced TRPA1-dependent mechanical allodynia. Present observations that constriction of the infraorbital nerve increased CCL2 levels within the injured area, and that both a systemic and perineural anti-CCL2 antibody attenuated monocyte/macrophage accumulation, hydrogen peroxide and 4-HNE increases, and nociception or hypersensitivity, indicate that local CCL2 release is a major contributing mechanism, most likely placed upstream to the cascade of cellular and molecular events that drive TRPA1-dependent pain-like behaviours (Fig. 8).

This study identifies for the first time the mechanisms that, from the original nerve insult, determine the pain-producing engagement of TRPA1 in a model of trigeminal neuropathic pain. However, a number of questions remain to be addressed, and some study limitations should be mentioned. While TRPA1 or oxidative stress blockade fully abrogated CION-evoked pain-like behaviours, CCL2 immunological inhibition and monocyte/macrophage depletion were associated with a substantial, but incomplete, attenuation of such responses. Residual effects could be due to inadequacy in terms of dosing or timing of the pharmacological interventions (CCL2 antibody and clodronate), or because additional cell type(s) and mediator(s) give a minor, but still meaningful, contribution to the overall phenomenon. Indeed, while CCL2, which is released by a variety of resident or inflammatory cells, seems to play a major role, it is possible that other chemokines (Old *et al.*, 2014) or additional pro-inflammatory mediators, upstream to CCL2, may contribute. Similarly, whereas pain-like

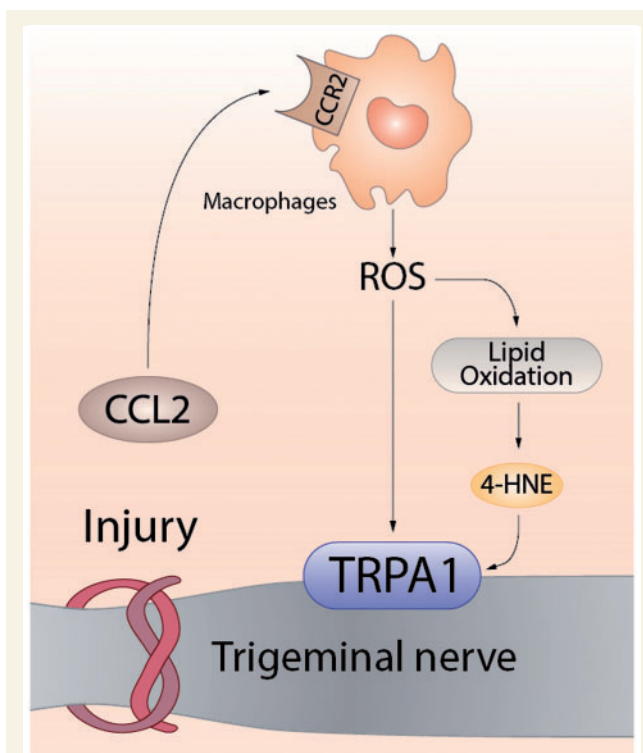


Figure 8 TRPA1-dependent mechanisms in the constriction of the infraorbital nerve. Drawing depicts the possible cellular and molecular events contributing to TRPA1-dependent pain-like behaviours induced by constriction of the infraorbital nerve. Surgery promotes, within the injured area, the release of CCL2, which, in turn, stimulates monocyte/macrophage recruitment and activation to generate oxidative stress (reactive oxygen species, ROS) and lipid peroxidation (4-HNE) byproducts, which engage TRPA1 in nociceptors, evoking pain-like behaviours.

behaviours seem mostly to depend on monocytes/macrophages, the contribution of additional pro-inflammatory cells cannot be ruled out (Vicuna *et al.*, 2015).

Trigeminal neuropathic pain affects a substantial proportion of the general population (Zakrzewska, 2013; Zakrzewska and Linskey, 2014) and patient treatment remains unsatisfactory (Renton *et al.*, 2012). Present findings that CCL2-dependent monocyte/macrophage accumulation and the ensuing oxidative stress by-products that engage TRPA1 are key factors for the development and maintenance of pain-like behaviours in a mouse model of trigeminal neuropathic pain offer a new interpretation of the pathophysiology of this condition. In this novel paradigm, different mechanisms identified in the present study emerge as new pharmacological targets for drug development. However, while predictable pharmacodynamic or pharmacokinetic hurdles may limit interventions directed to inhibit monocyte/macrophage accumulation or oxidative stress, TRPA1 blockade appears to be a feasible endeavor, fostered by the current clinical development of channel antagonists.

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